**In vitro Stimulation of Presensitized Mouse Spleen Cells with Poliovirus Type 1, Mahoney, and Enhancement of Poliovirus-specific Hybridomas**

By KLAUS-JOCHEN WIEGERS,* HANS UHLIG AND RUDOLF DERNICK

Heinrich-Pette-Institut für Experimentelle Virologie und Immunologie an der Universität Hamburg, Martinistrasse 52, 2000 Hamburg 20, F.R.G.

(Accepted 4 June 1986)

**SUMMARY**

*In vivo* immunization of BALB/c mice with poliovirus type 1, strain Mahoney, or with its purified polypeptides resulted in 0.2 to 0.5 antigen-specific hybridoma microcultures per $10^6$ spleen cells. Stimulation of spleen cells from mice immunized with poliovirus or with its polypeptides *in vitro* with poliovirus 6 days prior to fusion with the myeloma cells led to a six- to 20-fold increase in the number of positive microcultures, i.e. after stimulation the yield of poliovirus-specific hybridomas was up to 3-8 antigen-specific microcultures per $10^6$ spleen cells. The *in vitro* stimulation of spleen cells primed *in vivo* was demonstrated by the detection of poliovirus-specific antibody-producing cells 6 days after *in vitro* cultivation in the presence of poliovirus as antigen. Only spleen cells stimulated under these conditions *in vitro* gave rise to specific antibody-producing cells and yielded antigen-specific hybridomas after somatic hybridization.

The hybridization of spleen cells from immunized mice with plasmacytoma cells is a powerful tool for the generation of monoclonal antibodies (Köhler & Milstein, 1975). However, the yield of antigen-specific hybridomas after *in vivo* immunization is in the range of 0.1 to 0.5 per $10^6$ spleen cells or less. Recently, it has been demonstrated that either *in vitro* cultivation of spleen cells from immunized mice in the presence of antigen or adoptive transfer of spleen cells from immunized mice with an additional *in vivo* boost led to a tenfold increase in the number of antigen-specific hybridomas. It was suggested that both protocols increased the number of antigen-specific cells, whereas the total number of cells available for hybridization decreased (Fox *et al.*, 1981; Siraganian *et al.*, 1983). Various conditions for *in vitro* immunization have been outlined in a recent review in which it has also been pointed out that *in vitro* immunization can lead to a different antibody response compared to the *in vivo* response (Reading, 1982). *In vitro* induction of an anamnestic response in primed mouse spleen cells by foot-and-mouth disease virus (FMDV) has also been described recently (Collen *et al.*, 1984).

For the investigation of structural, functional and antigenic contributions of proteins VP1 and VP2 to the structure of the poliovirus particle and its precursors we wanted to raise a large panel of monoclonal antibodies against each polypeptide.

Poliovirus type 1, Mahoney, and naturally occurring empty capsids (NECs) were prepared according to standard procedures (Drzeniek & Bilello, 1974). Pure poliovirus polypeptides were obtained by reversed phase HPLC in 60% formic acid and a gradient of acetonitrile (Heukeshoven & Dernick, 1985). After freeze-drying they were dissolved in 8 M-urea and 0.1% dithioerythritol. BALB/c mice were used for immunization and the P3-NS-1-Ag4-1 (NS-1) myeloma cell line for the production of hybridomas. In our experiments we used two immunization protocols. (i) For *in vivo* immunization mice were primed subcutaneously with 20 μg of antigen, either with poliovirus or with its purified polypeptides in complete Freund's adjuvant. Four weeks later they were boosted intraperitoneally three times with 20 μg of antigen.
Fig. 1. In vitro induction of poliovirus-specific antibodies. Spleen cells from mice immunized with poliovirus, type 1, Mahoney (a, b) or with its purified VP1 (c) or VP2 (d) were assayed by ELISA for virus-specific antibodies either on day 0 (a) or day 6 (b, c, d) of cultivation in vitro in the absence (closed symbols) or presence (open symbols) of 5 µg/ml poliovirus. The ELISA plates were either coated with poliovirus (○, ●) or NECs (□, ▲) (5 µg/ml in phosphate-buffered saline pH 7.2; PBS) overnight at 4 °C. Plates were washed once with PBS. They were then blocked for 1 h at 37 °C with RPMI + 15% FCS. Control wells were only blocked. Then 0.1 ml of serial twofold dilutions of spleen cells starting from 5 × 10⁵ cells/ml were added to replicate wells and cultured overnight at 37 °C. After washing with PBS-Tween (0.05% Tween 80), they were incubated with rabbit anti-mouse immunoglobulins (Dako) diluted 1:100 in blocking solution (10% bovine serum in PBS) for 1 h at 37 °C. The plates were washed again with PBS-Tween and incubated for 2 h at 37 °C with peroxidase-labelled swine anti-rabbit immunoglobulins (Dako) diluted 1:500 in blocking solution. Results were measured at two wavelengths in a multiscan ELISA reader (Flow Laboratories) and expressed as differences of absorbance at 492 and 540 nm.

without adjuvant on three successive days and fusion was carried out on day 4 (Stähli et al., 1980). (ii) For in vitro cultivation mice were primed as above. They were boosted once with 20 µg of antigen after 4 weeks. After another 7 weeks the spleens were removed and spleen cells prepared. In vitro cultivation was either done in 9 cm Petri dishes (tissue culture grade, no. 632171; Greiner, Nürtlingen, F.R.G.) or in 80 cm² culture flasks (no. 1-53732, Nunc). Pooled spleen cells (4 × 10⁶/ml) were cultivated in RPMI 1640 (Boehringer) containing 15% foetal bovine serum (FCS, Gibco). The medium was supplemented according to Kennett et al. (1978) and contained 17% conditioned medium from the NS-1 myeloma cells. The spleen cells were
cultured in the presence of 5 μg/ml of poliovirus as antigen, without rocking or feeding, in a 37 °C incubator in a humid atmosphere containing 7% CO2 and 93% air. Control cultures were kept under identical conditions without poliovirus. The cells were collected on day 6 and their viability was determined by trypan blue exclusion. The proportion of viable cells recovered was usually in the range of 20 to 50%. Fusion was carried out at a viable nucleated cell to myeloma cell ratio of 5 : 1 (Kennett et al., 1978). The cells obtained were plated into 96-well tissue culture plates (no. 1-67008, Nunc) at a density of 2 × 10³ spleen cells/well. Hybridomas were selected in HAT medium (Kennett et al., 1978). Hybridoma supernatants were screened with an indirect solid-phase ELISA using either poliovirus or NECs as bound antigen (Uhlig et al., 1983). Irrelevant hybridoma supernatants produced against influenza virus served as controls.

The effect of in vitro stimulation was measured by specific antibody production by the spleen cells in an indirect solid-phase ELISA (Kelly et al., 1979; Boerrigter et al., 1983).

In vitro stimulation of primed spleen cells was first studied with cells from mice immunized with poliovirus. Virus-specific antibody production was detected on day 6 in those cells which were cultured in the presence of poliovirus (Fig. 1 b). The amount of virus-specific antibody detected depended on the number of spleen cells seeded into each well. No virus-specific antibodies could be detected on day 0, the day of the preparation of spleen cells (Fig. 1 a) and on day 6 in the control cultures without antigen (Fig. 1 b). Thus, the presence of poliovirus in these spleen cultures primed in vivo gave rise to virus-specific antibody-producing cells after 6 days of in vitro cultivation.

Similar experiments were then performed with spleen cells from mice which had been primed with purified poliovirus polypeptides VP1 or VP2. Each of these spleen cell populations was cultured in vitro in the presence or absence of poliovirus as antigen. Poliovirus-specific antibody production was measured on day 6. In this case, besides virus NECs were also used as bound antigen because they share more antigenic determinants with isolated polypeptides than virus (Wiegers & Dernick, 1985). In both spleen cell preparations primed either with VP1 (Fig. 1 c) or VP2 (Fig. 1 d) poliovirus-specific antibodies were detected regardless of whether virus or NECs were used as bound antigen in the ELISA. The amount depended on the number of spleen cells in the assay. In the cell cultures not stimulated with poliovirus, virus-specific antibodies could not be detected under these conditions. Thus, spleen cells from mice primed in vivo with viral polypeptides can also be triggered to antibody production during in vitro cultivation with poliovirus as antigen.

Since our main interest was the production of a large number of monoclonal antibodies against each polypeptide, the bulk of each spleen cell preparation primed with VP1 or VP2 was stimulated in vitro and fused with myeloma cells. For comparison spleen cells primed in vivo with virus and stimulated in vitro were also fused. Spleen cells from control cultures cultivated in the absence of poliovirus were also fused. The frequency of virus-specific hybridomas obtained is shown in Table 1.

---

**Table 1. Enhancement of poliovirus-specific hybridoma production**

<table>
<thead>
<tr>
<th>Expt. no.</th>
<th>Antigen</th>
<th>Days of culture</th>
<th>Recovery of spleen cells (%)*</th>
<th>No. of positive wells/total no.†</th>
<th>No. of positive wells per 10⁶ spleen cells†</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Virion/-</td>
<td>0</td>
<td>100</td>
<td>0/96</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>Virion/-</td>
<td>6</td>
<td>39</td>
<td>0/192</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>Virion/virion</td>
<td>6</td>
<td>56</td>
<td>74/384</td>
<td>3.4</td>
</tr>
<tr>
<td>4</td>
<td>VP1/-</td>
<td>6</td>
<td>17</td>
<td>0/192</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>VP1/virion</td>
<td>6</td>
<td>14</td>
<td>179/576</td>
<td>3.1</td>
</tr>
<tr>
<td>6</td>
<td>VP2/-</td>
<td>6</td>
<td>18</td>
<td>0/192</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>VP2/virion</td>
<td>6</td>
<td>30</td>
<td>458/576</td>
<td>3.8</td>
</tr>
</tbody>
</table>

* Determined by trypan blue exclusion.
† A₄₉₂ - A₅₄₀ at least fivefold above value obtained with irrelevant supernatant.
After in vitro cultivation in the presence of poliovirus a slightly higher number of viable spleen cells was recovered with the exception of experiment 5, where half of the spleen cells were lost due to some toxic material in a tissue culture flask (Table 1). Spleen cells primed in vitro with poliovirus and stimulated in vitro with the same antigen yielded 3-4 poliovirus-specific microcultures per 10^6 spleen cells. Similar ratios were also obtained with spleen cells primed in vivo with purified poliovirus polypeptides and stimulated in vitro with virus. After in vivo immunization only (protocol i) we obtained 0.2 to 0.5 positive microcultures per 10^6 spleen cells. It is generally accepted that in vivo immunization of mice yields about 0.1 antigen-specific hybridomas per 10^6 spleen cells (Van Meel et al., 1985). Thus, after in vitro stimulation of primed spleen cells (protocol ii) we obtained at least a six- to 20-fold increase in the yield of antigen-specific hybridomas. In contrast, fusion of spleen cells of hyperimmune animals even after an in vivo boost resulted in a marked reduction of antigen-specific hybridomas (Oi et al., 1978; our own observation, unpublished).

The majority of hybridomas (> 90%) obtained in experiment 3 (Table 1) produced antibodies specific for the serotype used for priming and in vitro stimulation, thus demonstrating the specificity of the stimulation. Hybridomas obtained after in vitro stimulation (experiments 5 and 7, Table 1) producing antibodies of the IgG class recognized both the priming polypeptide and the virion used for in vitro stimulation, thus representing a typical secondary response. However, IgM-secreting hybridomas were also found, and these produced antibodies which were specific for the stimulating virion and did not recognize the priming polypeptide in an immunoblot. Whether these hybridomas were the result of a true primary response in addition to the anamnestic response in our in vitro cultivation system is currently under investigation.

We thank M. Hilbrig and U. Kuck for excellent technical assistance and Dr J. Heukeshoven for the separation of viral polypeptides. The Heinrich-Pette-Institut is financially supported by Freie und Hansestadt Hamburg and Bundesministerium für Jugend, Familie und Gesundheit, Bonn.

REFERENCES


Kelly, B. S., Levy, J. G. & Sikora, L. (1979). The use of the enzyme-linked immunosorbent assay (ELISA) for the detection and quantification of specific antibody from cell cultures. Immunology 37, 45-52.


(Received 29 January 1986)